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1) **Baurle J, Grusser-Cornehls U. Calbindin D-28k in the lateral vestibular nucleus of mutant mice as a tool to reveal Purkinje cell plasticity.** Neurosci Lett. 1994 Feb 14;167(1-2):85-8.

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Calbindin D-28k in the lateral vestibular nucleus of mutant mice as a tool to reveal Purkinje cell plasticity

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Antibodies against the calcium-binding protein Calbindin D-28k (CaBP) are specific markers of cerebellar Purkinje cells (PC). To identify the origin of CaBP-immunopositive (CaBP+) terminals and fibres in the dorsal part of the lateral vestibular nucleus (dLVN), brains of Purkinje cell degeneration mutants (PCD) were immunoreacted for CaBP using the avidin–biotin method (ABC). In PCD an almost complete loss of CaBP+ fibres and terminals in the dLVN compared to the wildtype and the Weaver mutant was present. Morphometric analysis of CaBP+ synaptic terminals in the dLVN of adult Weaver mutants showed that the maximum and mean terminal size exceeded those in wildtypes by almost twice, which is a far larger difference than in GABA-immunoreacted material. The results show that CaBP-immunoreactivity and terminal size expansion in Weaver are both mainly attributable to PCs. Moreover, it can be concluded that the colocalization of CaBP and GABA in fibres and terminals of the dLVN in normal animals is almost entirely restricted to the PC-innervation of this nucleus. Therefore CaBP-immunocytochemistry is an excellent tool to selectively investigate the direct PC-projections in the dLVN, as it sets off the GABAergic PC-innervation from the total GABAergic innervation of this area.

The calcium-binding protein Calbindin D-28k (CaBP) is known to be expressed at high rates by all cerebellar Purkinje cells (PC) without exception from an early embryonic stage (E11) until adulthood (for review see ref. 3). As the whole output of the cerebellar cortex is transported exclusively via the PC, a large fraction of CaBP-immunopositive (CaBP+) elements in the terminal domains (i.e. the deep cerebellar and vestibular nuclei) of the cerebellum must be derived from PC. However, at present the origin of CaBP+ fibres and terminals is in most instances unknown in contrast to the extensive mapping of CaBP+ somata throughout the brain (for review see 1). In the present study we have attempted to solve the question of the origin of CaBP+ fibres and terminals in the dorsal part of the lateral vestibular nucleus (dLVN).

The PCD-mutant, in which a gene defect leads to a rapid and almost complete PC-degeneration postnatally [11] (less than 1% of PCs survive [14]), is a useful model for identifying the sources of CaBP+ axons and terminals in the dLVN, the most heavily innervated target of PC-projections outside the cerebellum [7]. Further, CaBP-immunocytochemistry in Weaver mutants (almost total absence of granule cells [10] and partial loss of Purkinje cells [4]) and size quantification of positive terminals in the dLVN should clarify whether the plastic processes (remodeling and terminal size enlargement) previously observed in GABA-immunoreacted sections of Weaver mutants [2] are also visible in CaBP-immunoreacted material. In addition, although colocalization studies of GABA and CaBP in the cerebellum exist [3], the origin of axons and terminals displaying both antigenicities in the dLVN has still to be determined.

For comparative immunocytochemical investigations, a total of 26 mice were used, the parents of which originated from the Jackson Laboratories (Bar Harbor, Maine, USA): 8 PCD-mutants (B6C3Fe *pcd/pcd*), 6 B6C3Fe-wildtypes (+/+), 6 Weaver mutants (B6CBA *wv/wv*) and 6 B6CBA-wildtypes (+/+). The Calbindin D-28k antibody used was a monoclonal mouse-IgG (Code-No. 300). Specification and potency of this clone have been fully characterized by various tests [6] and numerous studies (for review see ref. 1). Initially the animals received a lethal dose of chloral-hydrate (Merck, Darmstadt, No. 2425) (1.75 g/kg body weight). Perfusion started with normal saline (0.9%) in 0.067 M PO_4 buffer at pH 7.4 and was followed by the fixative containing 1%

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paraformaldehyde (Merck, Darmstadt, No. 4005) and 1% glutaraldehyde (Merck, Darmstadt, No. 4239) in 0.1 M PO_4 buffer at pH 7.4 for 15 min. After 2 h of postfixation in the same fixative, brains were cut in the coronal plane at 30 μm on a vibratome (TPI Series 1000). Immunocytochemistry was performed according to a standard protocol of the ABC-method [9]. In preliminary tests, various dilutions of the first antibody were screened to obtain optimal immunostaining; intense, Golgi-like staining of cerebellar PC was achieved with a dilution of 1:6000 of the CaBP antibody.

Terminal sizes were quantified in 6 pairs of coprocessed wildtypes (B6CBA) and Weaver mutants (see Fig. 4). Morphometric analysis was restricted to those terminals synapsing from underneath on the somatic surface of giant cells in the dLVN (see Fig. 3), thus allowing visualization of unsectioned terminals in their full diameter. A detailed description of the quantification method was given previously [2].

In the wildtypes B6CBA and B6C3Fe as well as in Weaver mutants, the dLVN is massively traversed and innervated by CaBP+ axons and terminals. The density of positive processes is to such a degree that the contours of the somata of innervated cells in this region are clearly visible (Fig. 1A,B). In contrast, all neuronal somata in the dLVN are immunonegative without preceding blockage of the axonal transport.

Regarding this region in PCD-mutants (Fig. 1C,D), an almost complete loss of immunopositive elements is present: a maximum of 2 or 3 terminals/cell remain and only very few CaBP+ fibres (arrowheads in Fig. 1D) are visible. The density of these fibres is lowest in the dorsocaudal part of the nucleus and increases slightly towards the dorsorostral. Thorough investigation of serial sections (Fig. 2) shows that most of these remaining fibres in PCD, if not all, originate from the VIII nerve and course precisely through the LVN, as has been established for primary vestibular afferents by previous studies [5,12]

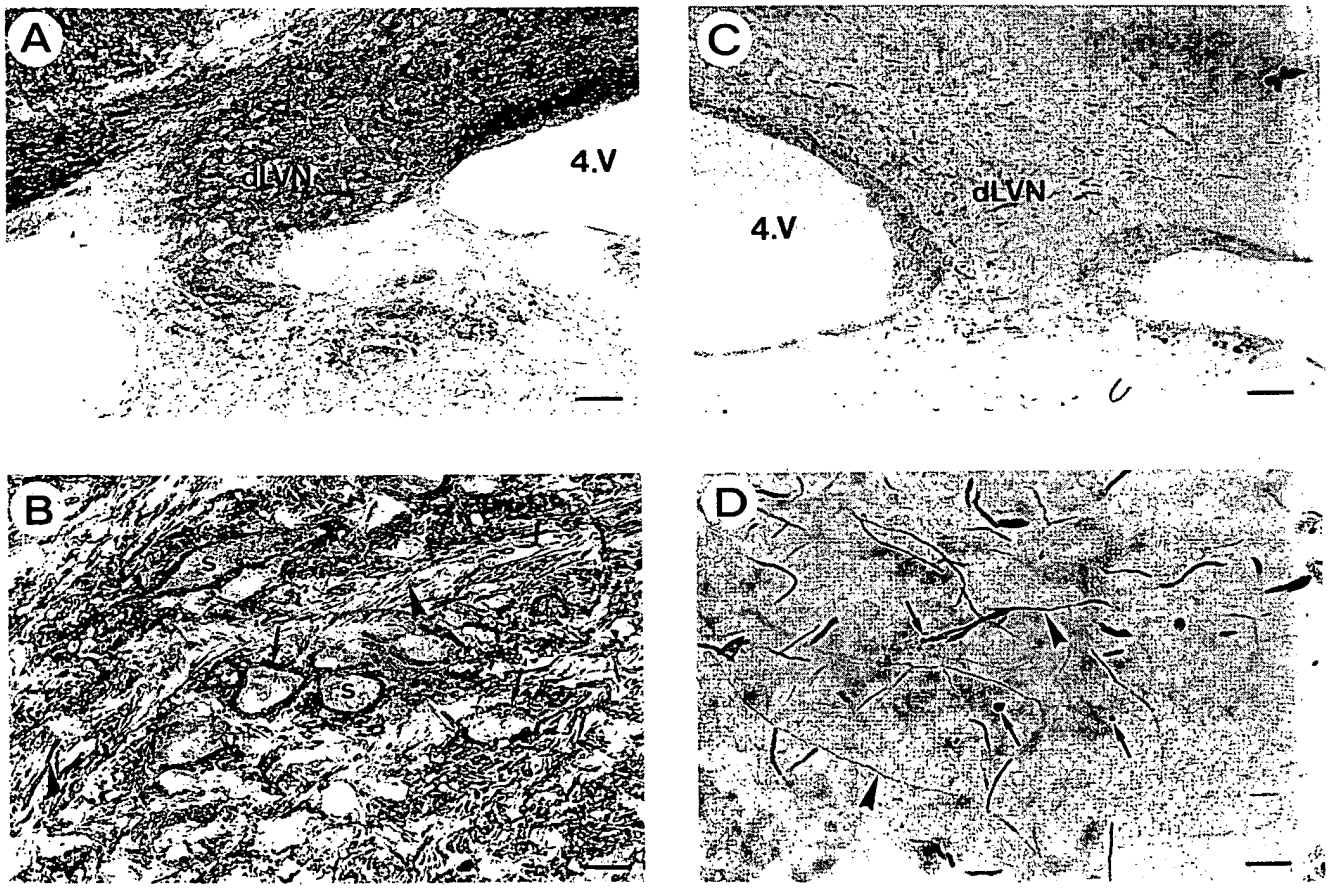
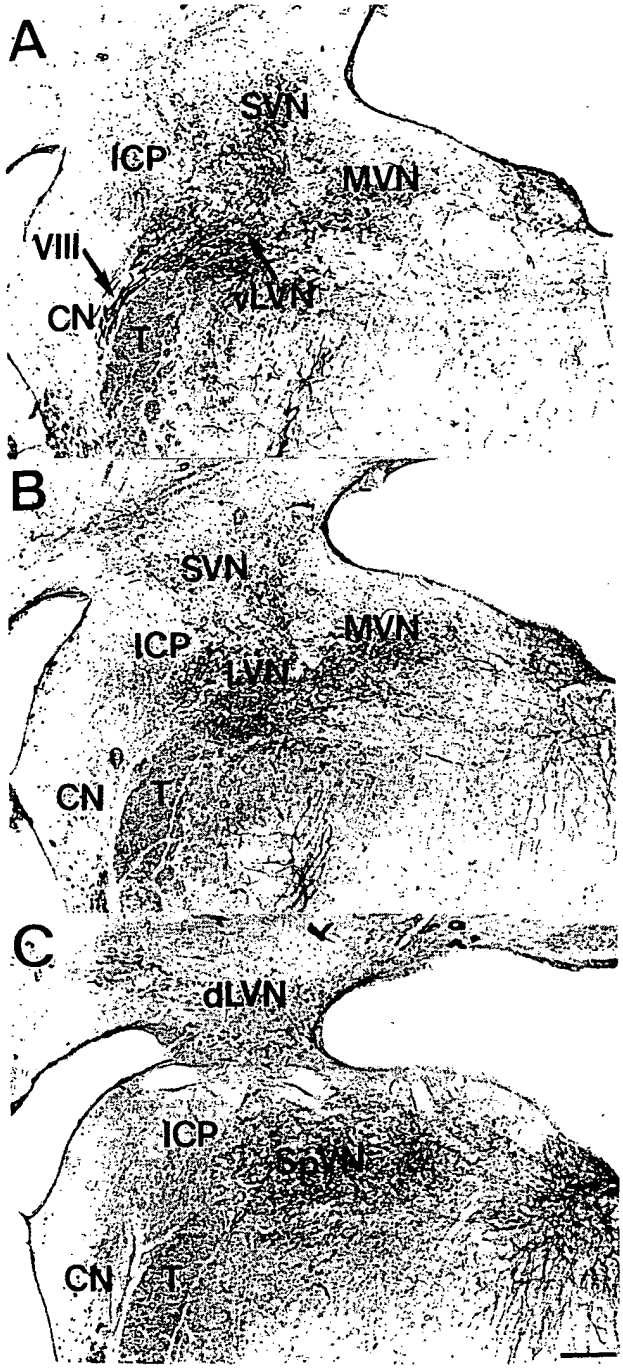


Fig. 1. Calbindin (CaBP)-immunoreactivity in the dorsal part of the lateral vestibular nucleus (dLVN) of normal (B6C3Fe-wildtype) and Purkinje cell degeneration (PCD) mice. Magnification: A,C = 63.4 ×, bar = 100 μm; B,D = 253 ×, bar = 25 μm. 4.V, fourth ventricle. A,B: B6C3Fe-wildtype. The dLVN is heavily innervated by CaBP+ elements. Neuronal somata (s) are unstained but the dense package of immunopositive terminals (arrows) make them visible. Fibres (arrowheads) which innervate or traverse the nucleus are present to a very high degree. C,D: PCD-mutant. In contrast to the wildtype, the Purkinje cell-deprivation in PCD-mutants causes a massive denervation of the dLVN, which leads to an almost complete loss of the CaBP+ fibres and terminals. Remaining immunopositive axons (arrowheads) of various diameters are thinly distributed through the neuropil, and CaBP+ positive terminal-like structures (arrows) are only found on rare occasions.

(see Fig. 2). However, it cannot be excluded that a very small proportion of fibres is of other origin. In normal animals and Weaver mutants, as shown in Fig. 1, the vast majority of CaBP+ elements in the dLVN are PC-processes. A high power view of CaBP+ terminals (Fig. 3) contacting immunonegative giant cells and size quantification of these terminals (Fig. 4) reveal a clear difference between wildtype and Weaver mutant. In the wildtypes,



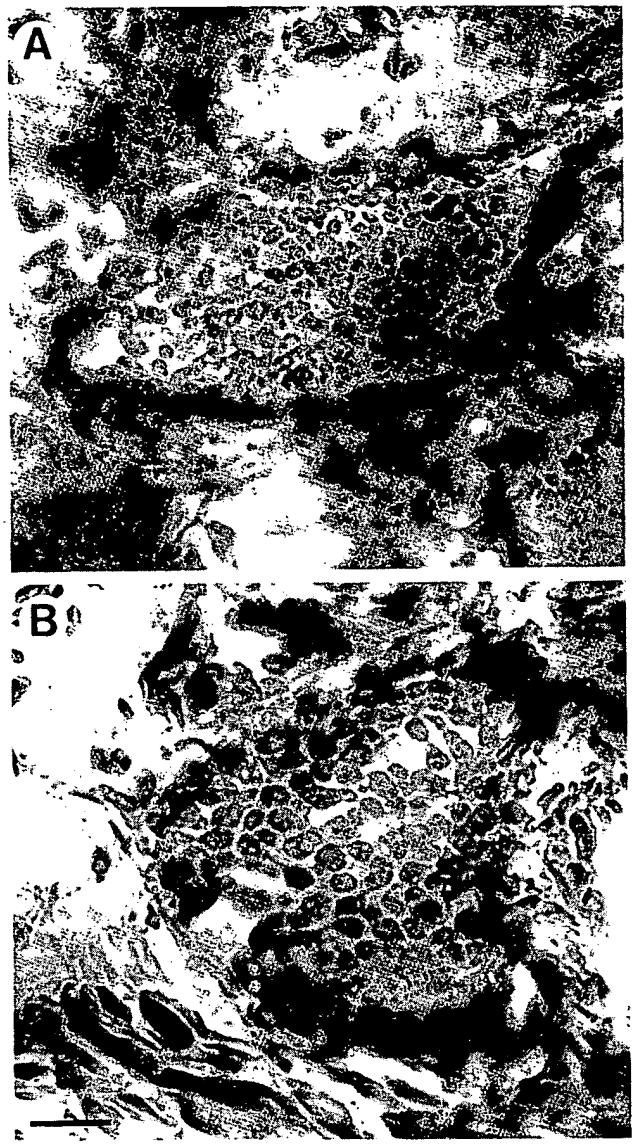


Fig. 3. Calbindin-immunopositive terminals synapsing on the somata of 'giant cells' in the dLVN of normal (A) (B6CBA-wildtype) and Weaver mutant mice (B). Magnification = 2028 ×, bar = 6.25 μm. Note the distinctly enlarged terminals in Weaver. Taking into account the results from PCD-mutants, these terminals must be derived from Purkinje cells.

Fig. 2. Distribution and extension of Calbindin-immunopositive (CaBP+) fibres in the vestibular nuclei of PCD-mutants. Sections A, B, C are aligned from rostral to caudal. Section thickness is 30 μm, every third section is shown. Magnification = 29 ×, bar = 250 μm. In panel A, the eighth nerve (VIII) enters the brainstem between the cochlear nuclei (CN) and the trigeminal nerve (T) and disperses its CaBP+ fibres massively into the most rostral portion of the ventral part of the lateral vestibular nucleus (vLVN). Two major branches extend beyond the LVN, one of them towards the superior vestibular nucleus (SVN), the other towards the medial vestibular nucleus (MVN). This is again visible in panel B. The highest density of fibres in the SVN is in the medial portion bordering on the MVN, but CaBP+ fibres reach the whole extension of the SVN. The MVN is innervated at a relatively high density only in its central portion. The further extension of the CaBP+ fibres is visible in panel C. The spinal vestibular nucleus (SpVN) is almost as heavily innervated as the vLVN, but the fibres reaching the caudal portion of the LVN, which is the dLVN, are at a relatively low density. ICP, inferior cerebellar peduncle.

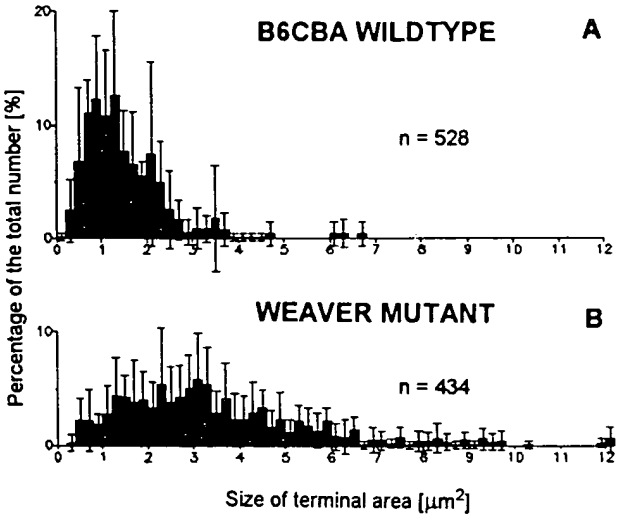


Fig. 4. Size distributions of Calbindin-immunopositive terminals in the dLVN of B6CBA-wildtype (A) and Weaver mutant (B) mice. The significance level (Wilcoxon) is at $P < 0.00005$ and the numbers of evaluated terminals are indicated. Highly significant differences in the distributions are obvious. Maximum and mean terminal sizes in Weaver are almost twice those in wildtypes.

the cross-sectional area of the largest terminals is at about 5 μm^2 and the peak of the size distribution is around 1 μm^2, whereas in Weaver mutants the largest terminals reach a size of up to 9 μm^2 and the peak is at about 3 μm^2.

The results presented in this study show that CaBP+ elements in the dLVN are nearly identical to the PC-innervation of this nucleus and that the differences in the size of CaBP+ terminals between wildtypes and Weaver mutants can therefore be attributed to those originating from PCs. They also clarify the extent of the colocalization of CaBP and GABA in this area. From recent studies it is known that a considerable non-PC-GABAergic innervation of this nucleus exists [2,8,14]. With regards to the present results, this GABAergic fraction must be CaBP negative, and therefore the colocalization of CaBP and GABA is restricted to the PC-GABAergic innervation. Axons and terminals from collaterals of traversing primary vestibular afferents in the dLVN are reported to be non-GABAergic (ref. 13, and own observations in our GABA-immunoreacted sections). In addition, since the degree of the terminal expansion in Weaver mice is far more pronounced in material immunoreacted for CaBP than for GABA [2], we conclude that it is mainly the PC-GABAergic fraction which is responsible for the size changes.

In conclusion, the results presented here demonstrate that CaBP-immunocytochemistry is a superior and highly selective method for marking and identifying PCs not only in the cerebellum but also their axons and terminals in the dLVN. The presence of plastic changes previously observed in anti-GABA-immunoreacted material is further evidenced and shown to be a property of Purkinje cells. For these reasons and with a view to further investigations, the dLVN of Weaver mutants can be considered as an easily accessible system in which plastic changes of identified neurons occur.

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